THE EFFECT OF PALMITOYLATION ON RGS10 SUBCELLULAR LOCALIZATION AND MEDIATING INFLAMMATION

By

POOJA MOHANTY

(Under the Direction of Shelley Hooks)

ABSTRACT

The canonical function of RGS10 (regulator of G-protein signaling) is to terminate G-protein activity by accelerating GTPase activity on the Gα subunit. Previous research has shown that under inflammatory conditions, RGS10 can suppress inflammatory cytokines. However, this function of RGS10 is G-protein independent. To investigate whether inflammation changes the subcellular localization of RGS10, a palmitoylation deficient RGS10 mutant (RGS10 C74A) was created. Palmitoylation is a post translational modification that enhances RGS10 association with the cell membrane. It was found that under inflammatory conditions, both wild-type RGS10 and RGS10 C74A exhibited higher protein expression than empty vector control at the membranes and nucleoplasm. Furthermore, there was no significant difference between wild-type RGS10 and RGS10 C74A in suppressing the inflammatory cytokine TNFα. It was concluded that palmitoylation is not required for RGS10's subcellular localization or function in suppressing inflammation. Further investigation is needed to understand the role of RGS10 in the nucleoplasm.

INDEX WORDS: Regulator of G-protein Signaling, Inflammation, Palmitoylation

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DEDICATION

I would like to dedicate this manuscript to my family, friends, and mentors. Without your love, support and encouragement, I would not be able to complete this journey alone.

To my fiancé, Deep Datta Roy, your positive energy and patience is incomparable. Thank you so much for always having my back and being the sound of reason.

To my parents, Dr. Bijoy K. Mohanty and Mrs. Bijaya Mohanty, and my sister, Neha Mohanty, thank you for your resolute support for all my educational endeavors.

To my friends, it has been a fun ride; we have finally made it.

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I would like to thank my parents and my fiancé for their continued support and encouragement of pursuing my dream. Earning this master's degree would not have been possible without you.

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And finally, I would like to thank all my lab members and the friends I have made along the way. It was fun embarking on this educational endeavor with you guys. I learned a lot from you and hope one day I will be able to return the favor.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 G-protein coupled receptors (GPCR)

GPCRs encompass the largest and most diverse group of transmembrane receptors in eukaryotes[1].They are heavily implicated in a wide variety of cellular signaling pathways and have been associated with an array of diseases, such as Alzheimer's [2, 3], ovarian cancer [4, 5], inflammation[6, 7] and multiple sclerosis[8]. Due to their diverse function and implications in many pathologies, GPCRs and their associated downstream counterparts are common drug targets.

GPCRS have seven transmembrane domains. Ligand binding occurs along the transmembrane loops exposed at the extracellular surface. Upon the binding of a ligand, the transmembrane protein undergoes a conformational change and initiates signal transduction on the cytoplasmic side[9, 10]. G-proteins that typically associate with GPCRs are heterotrimeric and have the ability to bind to guanosine triphosphate (GTP) and guanosine diphosphate (GDP). These heterotrimeric G-proteins are composed of three subunits: α,β, and γ. Gα has four major subfamilies: i, s, q and 12. Gαs stimulate adenylate cyclase (AC), which increases the production of cyclic adenosine monophosphate (cAMP), a downstream messenger molecule. Activation of GPCR confers it as a guanine-nucleotide exchange factor (GEF) which promotes the exchange of the bound GDP for GTP[11, 12]. Conversely, Gαi inhibits AC and decreases the production of cAMP. Gαq couples to phospholipase C and increases the intracellular concentration of calcium. Gα12 activity affects actin remodeling in cells and is a part of the RhoGEF pathway [13].

G-proteins have intrinsic GTPase activity which helps them deactivate by hydrolyzing GTP to GDP [14]. However, the k_{cat} of this reaction is 5 min^{-1}, a very slow kinetic process overall [15]. Thus, another protein which can accelerate the rate of hydrolysis, such as RGS proteins, is needed to terminate GPCR activity.

1.2. Regulator of G proteins Signaling (RGS)

RGS proteins are a class of proteins which act on the Gα subunit and accelerate the rate of GTP hydrolysis[16]. Structurally, RGS is comprised on 9 alpha helixes which bind to the transition state of Gα with three critical contact points and work as a GTPase accelerating protein (GAP)[17, 18]. It has been observed that RGS proteins demonstrate specificity for the Gαi subunit [19]. To date, there have been more than thirty RGS proteins identified, defined by their conserved RGS domain. They are subdivided into families depending on structural similarity and the presence of accessory domains outside the RGS domain [20, 21]. Traditionally, RGS proteins bind to the GTPase moiety associated with the Gα subunit and lower the energy of activation for the hydrolysis of GTP[18, 22] . They do not have any intrinsic catalytic activity but instead facilitate an environment which is favorable to lower the activation energy for GTP hydrolysis[22]. It was once believed that RGS proteins only negatively regulate GPCR signaling but recent data has shown that RGS interaction with the GPCR is complex and results in an array of downstream signaling (**Figure 1)**. Considerable evidence has also shown that non-RGS domains also interact with the GPCR and carry out functions that are distinct from GPCR signaling[21, 23].

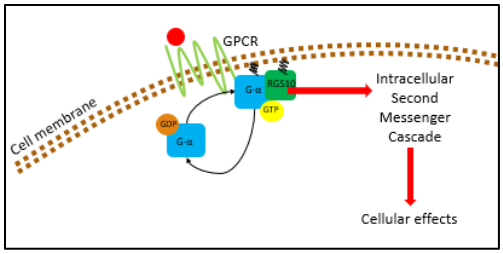

Fig 1.RGS10 function. The function of RGS10 interaction with the Gα subunit terminates G-protein signaling**.**

The RGS protein superfamily is relatively small in molecular size and highly conserved in the RGS domain (120 amino acids) **(Figure 2)** [24]. The Hook's lab is particularly interested in RGS10, a member of the R12 family (fig)[25] and its role in modulating inflammation and cancer resistance.

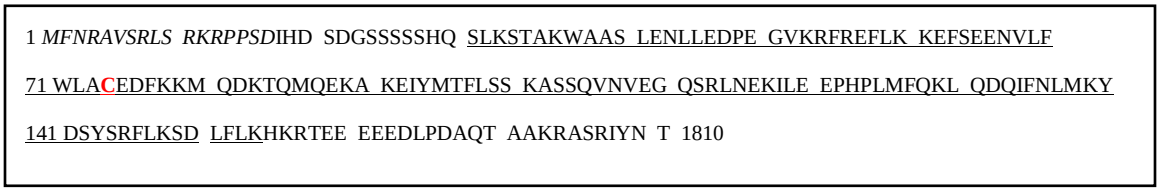

Fig 2. RGS10 amino acid sequence. The conserved RGS 10 domain is underlined. The site of palmitoylation (C74) is in red.

1.3 RGS10 and inflammation

Microglia perform as immune cells for the central nervous system and are the primary source of inflammation in the brain [26]. Upon insult or injury (such as infections, toxins or other external stimuli), microglia become activated [27] and release inflammatory signals in the form of cytokines (i.e. TNFα, IL-1β) [26, 28-30]. It has been predicted that the production of these inflammatory mediators may act as a quick recruiting signal for peripheral immune cells into the central nervous system to destroy invading antigens [26]. However, prolonged inflammation can lead to damage of other cells in the central nervous system, such as neurons, that may lead to deleterious effects for the central nervous system. RGS10 has been identified in a host of cells and has been shown to play a pivotal role in modulating the inflammatory response in microglia [31].

Canonically, RGS10 works to facilitate the transition from GTP to GDP[27]. Under inflammatory conditions, it has been observed that RGS10 expression is suppressed in comparison to non-inflammatory conditions[32]. A recent study conducted by the Hooks lab demonstrated RGS10 may not be regulating inflammation through a canonical manner. For example, when G-protein:RGS10 binding was hindered, TNFα levels remained low during normal physiological conditions indicating RGS10 is still downregulating TNFα levels without the help of Gαi. Furthermore, suppression of RGS10 enhanced TNFα and COX-2 without GPCR activity[33] indicating G-protein:RGS10 binding is not necessary for regulation of TNFα . Understanding whether the subcellular localization of RGS10 under inflammatory and non-inflammatory conditions could better explain how RGS10 is regulating inflammation.

1.4. RGS10 Localization

RGS proteins are expressed throughout many body tissues but are most abundant in the thymus, spleen, hippocampus, striatum, and neocortex [34]. Their attachment to the phospholipid membrane is thought to be modulated via palmitoylation of a cysteine within the conserved RGS domain among many different subtypes of RGS proteins [35]. However, the function of RGS10 at the cell membrane has not been clearly understood [17, 25, 36, 37].

The expression level and subcellular localization of RGS can be vastly differently based on the isoform or cell type. RGS subcellular expression can vary as well. For example, a study conducted by Chatterjee and Fisher (2000), showed that RGS2 was mainly found in the nucleus while RGS4 was mainly cytoplasmic in COS-7 cells. In the same cell line, RGS10 was predominantly nuclear while RGS16 was found in both the nucleus and cytoplasm. Cytoplasmic RGS proteins travel to the membrane, presumably following Gαi signaling[38]. The role of RGS in the nucleus remains unknown but it has been a key area of research. In RGS4 and 16, there is a nuclear export sequence (NES) on the N-terminus which promotes the export via exportin 1 located on the nuclear envelope [38]. In RGS10, protein kinase A (PKA) has been shown to phosphorylate serine 168 which mediates translocation of RGS from the cytosol to the nucleus [39, 40].

It has been recently discovered that RGS10 may be playing a neuroprotective role in microglia. A study by Lee et al (2011) demonstrated that microglia treated with lipopolysaccharide (LPS), an inflammatory endotoxin, resulted in higher RG10 protein expression in the nucleus [41]. They found that phosphorylation of a conserved serine within the RGS domain leads to the nuclear import of RGS10. This group concluded that RGS10 may be playing a non-traditional role in the

nucleus where RGS10 may be modulating the NF-κB pathway. However, the mode of activation was unclear.

1.5 Palmitoylation in RGS proteins

Palmitoylation is a post-translational modification which occurs when thioester groups on cysteine residues are modified by protein-acetyltransferase with the addition of one or more palmitoyl groups.[42](**Figure 3).** It plays two roles in the cell: as a tether to the membrane and/or a marker for lipid raft identification. This form of modification is one many forms of lipid modification a protein can undergo including myristolyation (addition of a 14 carbon myristic group) and prenylation (addition of a 15 carbon prenoid group) [43]. In comparison, palmitoylation is a 16-carbon palmitic acid addition. The presence of the extra carbons in palmitoylation is believed to increase the membrane affinity, one of its major functions, of the protein to a point where translocation from the membrane is strongly inhibited unless the lipid moiety is removed[44]. Another key difference between palmitoylation and other forms of lipid modification is its reversibility. Unlike myristolyation and prenylation, palmitic acid can be added or removed from the molecule throughout its life [45] and can occur in conjunction with other lipid modifications [46]. The reversibility of the palmitoylation plays an important role in protein localization within different compartments in the cell [47]. There are four distinctive types of palmitoylation : (1) a single palmitoyl modification frequently near the end of a protein; (2) palmitoylation proximal to a transmembrane domain; (3) dual palmitoylation and prenylation; and (4) dual palmitoylation and myristolyation[48, 49]

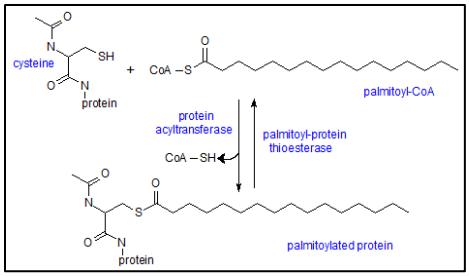

Fig 3. Palmitoylation in RGS10. It is a reversible process by which a cysteine residue on the proteins is modified by the addition palmitoyl-CoA by palmitoyl-protein thioesterase

In RGS proteins, the palmitoylated state favors interaction with the Gα subunit[50] and affects GAP activity in some subtypes [51-53]. Due to the large variation in RGS protein structures, lipid modification occurs at different sites on any given RGS protein. For example, palmitoylation occurs at the amino terminus in RGS2 and 3 [54] while RGS6 and 7 are palmitoylated within the RGS domain[55]. Similar to many of its counterparts in the R12 family, RGS10 is palmitoylated within the RGS domain. In RGS10, there is one known palmitoylation site within this domain. [35, 54]. In the RGS10-2 isoform used in this study, C74 was the site of palmitoylation (**Figure 2)**.

The role of palmitoylation in RGS10 has largely been debated. While some have shown that this lipid modification enhances RGS10 GAP activity[35, 54], others propose that palmitoylation hinders GAP activity[35]. For example, a study conducted by Castro-Fernandez et al (2002) demonstrated that mutation of the conserved C60 residue in RGS10 negated its GAP function indicating palmitoylation of the conserved C60 is essential for RGS10 GAP function. Similarly, a study conducted by Tu et al (1999) demonstrated higher GTP hydrolysis in phospholipid vesicles containing Gαi seeded with palmitoylated RGS10 versus non-palmitoylated RGS10. This study indicated that the presence of palmitoylation facilitated the GTP hydrolysis function in RGS10. But in the same study, Tu et al also demonstrated that in a solution-based assay, RGS10's GAP activity diminishes as it takes up palmityl-CoA. Thus, the role of palmitoylation in RGS10 is largely unknown. Furthermore, the effect of palmitoylation on RGS10 localization has not been clearly defined. Thus, understanding how palmitoylation affects RGS10 activity and localization can give us a better insight as to the function RGS10 in inflammation and other diseases.

To answer these questions, the goal of this study was to identify the localization and effect of wild-type RGS10 and RGS10-C74A (palmitoylation deficient RGS10) under resting and inflammatory conditions. Understanding the localization under these two conditions would shed light upon whether translocation occurs upon inflammation in RGS10 and whether palmitoylation is needed for RGS10 translocation. Past research has demonstrated that wild-type RGS10 can suppress TNFα during inflammation[31]. This assay has not been conducted in RGS10 C75A and can potentially uncover a functional aspect of palmitoylation.

CHPATER 2

MATERIALS AND METHODS

2.1. Cells and Regents

The murine BV-2 microglia cell line was a gift from G. Hasko at the University of Medicine and Dentistry of New Jersey and was previously generated by Blasi et al. (1990). BV-2 cells were maintained in Dulbecco's modified Eagle's medium (VWR) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific). The HEK-Blue hTLR4 cell line was purchased from InvivoGen and was maintained in Dulbecco's modified Eagle's medium (VWR) with low-endotoxin 10% fetal bovine serum and HEK-Blue Selection antibiotics (InvivoGen) to selectively maintain cells overexpressing TLR4 and adapter proteins. Lipopolysaccharide (LPS) was obtained from Sigma-Aldrich.

2.2. RGS10 C74A mutation propagation

Site directed mutagenesis kit (ThermoFisher) was used to create the palmitoylation deficient RGS10 mutant by replacing C74 with an alanine. This was done by using the following C74A primer sequence: Forward: 5' GTT TTG TTT TGG CTA GCA GCT GAA GAT TTT AAG AAA ATG- 3'; reverse: 5- CAT TTT CTT AAA ATC TTC AGC TGC TAG CCA AAA CAA AAA-3'. Polymerase chain reaction was then used to amply this gene of interest before it was transformed into XL1-BLue competent cells (Stratagene). RGS10 C74A plasmid was

collected using a maxi prep kit purchased form E.Z.N.A and the site of mutation was confirmed using sanger sequencing (UGA core facility) **(Figure 4**). The purified plasmid was labeled and stored at -20 C until experimentation.

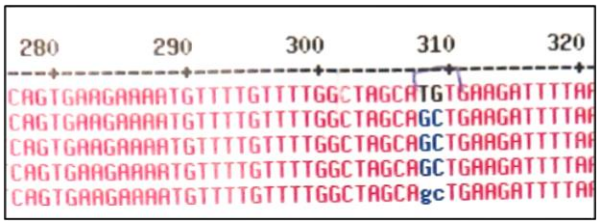

Figure 4. Sanger sequencing data showing successful point mutation from cysteine (TGT) to alanine (GCT)

2.3. **Plasmid Transfection**

Transfection was conducted using lipofectamine/PLUS regent according to the manufacturer's instructions (Thermo Fisher). 2 µg of DNA was added per well in a 12 well plate or scaled according to the plate or well size. Cells were cultured for 24 hours in antibiotic free medium prior to experimentation.

Cells used for western blotting were harvested in SDS-page lysis buffer. Cells harvested for RT-PCR were harvested in Trizol. Cells harvested for fractionation were harvested in PBS supplemented with protease inhibitors.

2.4 Quantitative Real-Time Polymerase Chain Reaction

mRNA was isolated using Trizol reagent (Invitrogen/Life Technologies) and cDNA was synthesized from 2 µg total RNA using High Capacity Reverse Transcriptase cDNA kit (Life Technologies/Thermo Fisher Scientific). Quantitative real-time polymerase chain reaction (PCR) was performed using Superscript III kit for RT-PCR (Invitrogen/Life Technologies) and Power SYBR Green reagent (Life Technologies/Thermo Fisher Scientific). Reactions were normalized using the housekeeping gene actin and $2^{-\Delta\Delta CT}$ method was used to perform the calculations. Actin primer sequence is as follows: Forward 5'- GGCTGTATTCCCCTCCATCG-3'; Reverse 3'-CCAGTTGGTAACAATGCCATGT-5'

2.5 Sucrose-based subcellular protein fractionation

A sucrose/HME extraction solution containing HME buffer (0.1 M Hepes, 1 M $MgCl_2$, 10% EDTA) and 12% sucrose (w/v) with added protease/phosphatase inhibitors was used. Cells were grown to 100% confluency on 150mm tissue culture dishes. They were washed with ice cold PBS and harvested using the sucrose/HME solution. The cells were manually homogenized

using a size A dounce apparatus. A sample was collected and set aside as “total cell lysate”. The homogenate was then centrifuged at 5,000g. The pellet was set aside as the “nuclear fraction”. A small portion of the supernatant as set aside as the “post nuclear lysate”. The remaining supernatant was then ultracentrafuged at 40,000g to isolate the “soluble” and “membrane” fractions. All fractions were resuspended in sucrose/HME buffer and stored at -20C until experimentation **(Figure 5)**

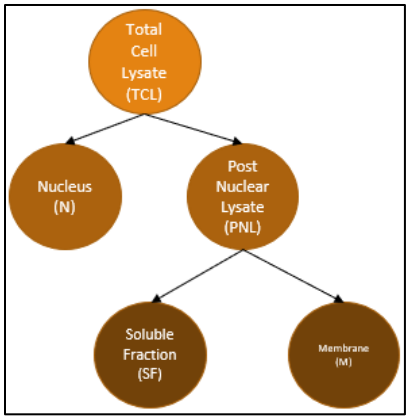

Figure 5. A step-wise breakdown of the sucrose-based subcellular fractionation.

2.6 Chemical gradient subcellular protein fractional

A subcellular protein fractionation kit wit was purchased from ThermoScinetific. All buffers listed below were included in the kit. The fractionation protocol was optimized as described (**Figure 6**):

Cells were grown to confluency in 12 well plates. They were harvested in ice-cold PBS treated with protease/phosphatase inhibitors. The fractionation was conducted on a 10 µl cell pellet isolated from the total cell lysate. The pellet was first treated with Cytoplasmic Extraction Buffer (CEB) and shaken gently at 4°C for 15 minutes after which it was centrifuged at 1000g. The supernatant was kept aside as the cytoplasmic fraction. The remaining pellet was treated with membrane extraction buffer (MEB) and shaken at RT for 10 minutes after which it was centrifuged at 3000g. The supernatant was kept aside as the membrane fraction. The remaining pellet was treated with nuclear extraction buffer (NEB) and shaken gently at 4°C for 30 minutes after which it was centrifuged at 5000g. The supernatant was kept as the soluble nuclear fraction. The remaining pellet was treated with NEB, calcium chloride and micrcoceal nuclease and incubated at 37°C for 5 minutes after which it was spun down at 16000g. The supernatant was extracted and kept aside as the insoluble nuclear lysate. The remaining pellet was discarded. The extracted fractions were immediately frozen down in sample buffer (2% SDS buffer).

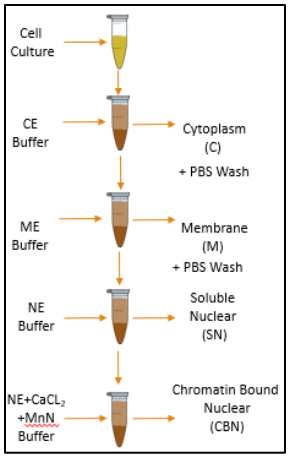

Figure 6: A step-wise breakdown of the chemical gradient subcellular fractionation.

2.7 Western Blotting

Evaluation of protein expression after subcellular protein fractionation was done by first normalizing protein concentration using BCA analysis kit (ThermoFisher) in accordance to the provided instructions. Each sample was then denatured by boiling for 5 minutes and analyzed using SDS-PAGE. Membranes were blocked with milk for one hour and incubated with primary antibodies overnight, washed and incubated with appropriate HRP-conjugated goat, rabbit, mouse secondary antibodies for one hour and imaged to visualize the proteins using enhanced chemiluminescence reagent (Thermo Fisher Scientific; Pierce). Primary antibodies for anti-RGS10, anti-H3 and anti-SP1 were purchased from Santa Cruz Biotechnologies. Anti-GAPDH antibody was purchased through Millipore Technologies. Anti-sodium/potassium antibody (a6F) was purchased through the Developmental Studies Hybridoma Bank.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. RGS10 C74A mutant expression in HEK cells

Wildtype RGS10 and RGS10 C74A plasmids were transfected in HEK cells to check for overexpression. As can be seen from **Figure 7A**, the expression of the RGS10 C74A is less than the wild type when the same amount of DNA was transfected. This result confirmed that RGS10 C74A could be over-expressed in-vitro. However, it also suggested that more RGS10 C74A needed to be transfected to equalize wild type RGS10 expression. This result was repeated and optimized using twice as much RGS10 C74A DNA (**Figure 7B**).

Low transfection efficiency in the RGS10 C74A mutant can be a result of its inability to form a DNA-lipid complex with the transfection reagents. It could also be due to the fact the cell may not be able to express the RGS10 C74A DNA as efficiently as the wildtype RGS10 due to impure plasmid quality or stability.

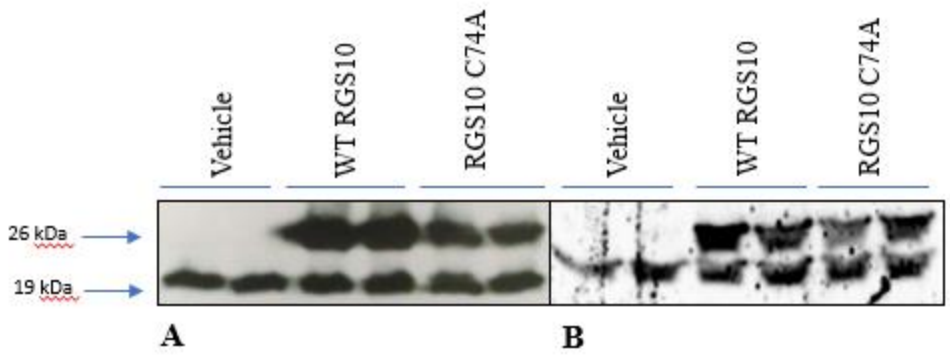

Figure 7. RGS10 expression in HEK cells A. Wild type RGS10 and RGS10 C74A overexpressed in HEK cells. **B.** Optimization of RGS10 C74A transfection

3.2. Sucrose based fractionation

A sucrose based fractional protocol was initially utilized to obtain subcellular fractions. Using this method, the total cell lysate, nuclear, post nuclear, cytoplasmic and membrane fractions were isolated. In order to verify that proper extraction had occurred with minimal cross compartmental contamination, GAPDH (Cytoplasm), Gβ-M14 (membrane), Total histone (nuclear) were used as internal controls. (**Figure 8)**

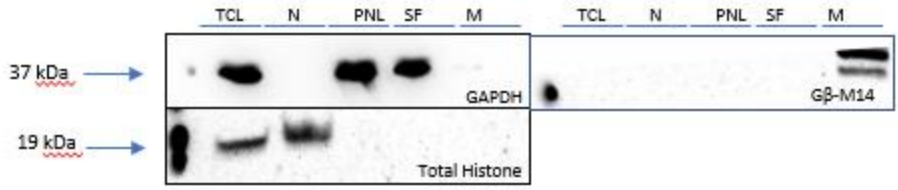

Figure 8. Internal controls: GAPDH-cytoplasm; Gβ-M14-membrane; Total histone-nuclear for sucrose-based fractionation.

However, a consistent and reproducible RGS10 banding pattern could not be obtained using this method. For example, in **Figure 9A** RGS10 is located in the nucleus, post nuclear lysate, soluble fraction and membrane fractions in HEK cells after a two-hour LPS (10 µg/mL) treatment. However, when this experiment was repeated with the exact same parameters, RGS10 was found to be located in the post nuclear lysate and soluble fraction **(Figure 9B).** This inconsistency may have been due to the physical homogenizing needed after each fraction isolation leading to deteriorated proteins causing results to be less accurate and less sensitive. Also, a large quantity of cells (~2mL packed cell volume) was needed for this protocol. Processing the cell lysates after each step was a time- consuming procedure which may have led to the denaturation of proteins. Thus, a faster and more sensitive method of protein fractionation was identified.

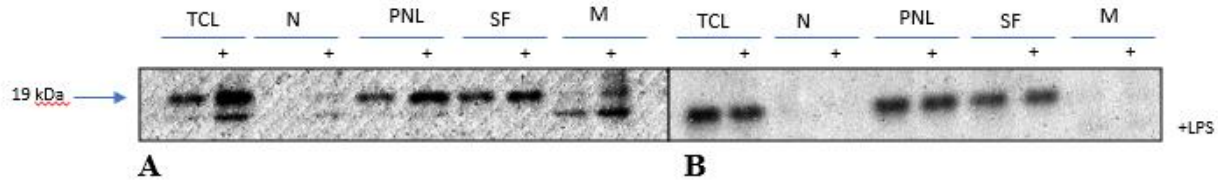

Figure 9. A-B inconsistencies in RGS10 banding patterns using a sucrose-based fractionation.

A chemical based fractional protocol yielded in quicker and more sensitive extraction method. Less cells were needed (~20 µL packed cell volume) eliminating a long processing time. Furthermore, physical homogenizing was not needed using this method. Each fraction was soluble in the given buffers eliminating protein deterioration due to physical homogenizing. This method also allowed the breakdown of the nucleus into two distinct compartments, the soluble nuclear lysate and the chromatin-bound lysate. This allowed for more sensitivity in the results. The antibody, SP1 was used as a marker for the soluble nuclear lysate. Furthermore, Na/K ATPase antibody was used instead of Gβ-M14 due to its strong association with the cell membrane (**Figure 10**).

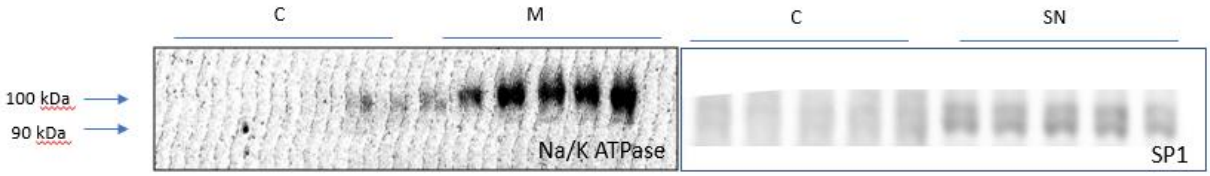

Figure 10. Internal control optimization. Na/K ATPase and SP1 were used as internal controls for the membrane and soluble nuclear fractions (respectively) for the chemical-based fractionation. GAPDH and total histone were used as cytoplasmic and chromatin bound fractions, respectively.

However, there were some shortcomings to this method as well. Since a smaller packed cell volume was needed, the overall protein concentration was lower. As a result, protein expression was harder to detect during western blotting. Furthermore, SP1 expression was not consistent in all of the fractionation. But this may have been a result of poor antibody quality and not reflective of the fractionation itself. Although this method was quicker than the sucrose-based assay, the efficiency of this method was based on the ability of the cell pellets to solubilize in the given buffers and the complete removal of each buffer after isolating a particular fraction. For example, if the pellet was not fully suspended in the buffer or pellet was not completely dry after each fractionation, inter-fraction contamination resulted. It was found that incubating the pellet in the cytoplasmic buffer for 15 decreased inter-fraction contamination **(Figure 11).**

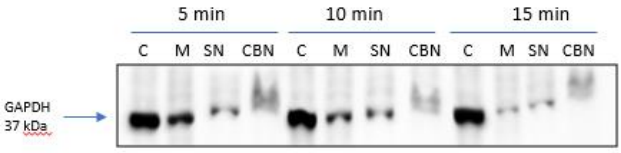

Figure 11. Different incubation times in cytoplasmic buffer. Optimal incubation time was determined to be at 15 minutes.

3. 3. Effect of LPS on RGS10 translocation in HEK cells

LPS is a ligand for toll like receptors (TLR). Activation of TLR receptors have shown to cause translocation of RGS10 to the nucleus, presumably through PKA signaling (Lee et al 2008). The purpose of this experiment was to establish an optimal dose and time of LPS treatment. To stimulate RGS10 translocation, HEK cells were treated with increasing concentrations of LPS from 10 µg/mL to 100010 µg/mL for either 2hr or 24 hours. The result of this assay suggests that increasing the dose of LPS has no effect on RGS10 translocation (**Figure 12**). In fact, higher doses of LPS resulted in toxicity in the HEK cells leading to as smaller viable cell volume for the assay. Thus, the smallest dose (10µg/mL) was used throughout the project in order to prevent toxicity. Likewise, LPS incubation time did not affect RGS10 translocation either. Furthermore, a study by Tu et al (1997) suggested that it takes ~2 hours for palmitoylation to occur in RGS10. Thus, to ensure palmitoylation occurs, a minimum of two hours of LPS incubation was used in the proceeding experiments.

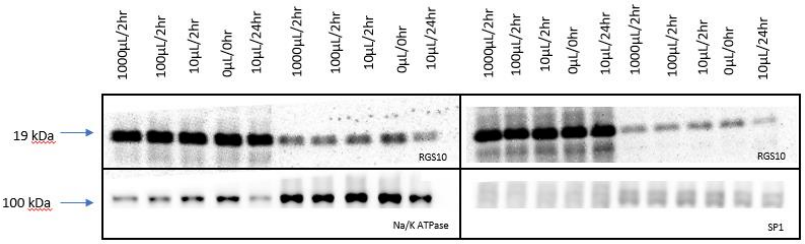

Figure 12. LPS dosage and incubation. Different LPS dosage or incubation time did not affect RGS10 translocation in HEK cells.

3.4. Endogenous RGS10 subcellular expression in BV2 cells

BV2 are microglial cells and present a physiologically relevant model for neuroinflammation. Two hour treatment of LPS (10 µg/mL) and subsequent subcellular fractions revealed that endogenous RGS10 is primarily located in the cytoplasm with little expression in the membrane and soluble nuclear lysate (**Figure 13**). Upon LPS stimulation, a decrease or increase in RGS10 expression in any of the subcellular compartments was not observed Thus, it was concluded that endogenous RGS10 in BV2 is not affected by LPS stimulation.

Over expression of wild type RGS10 and RGS10 C74A was attempted in the BV2 line as well but due to the high concentration of endogenous RGS10 already present in this cell line, over expression was not possible.

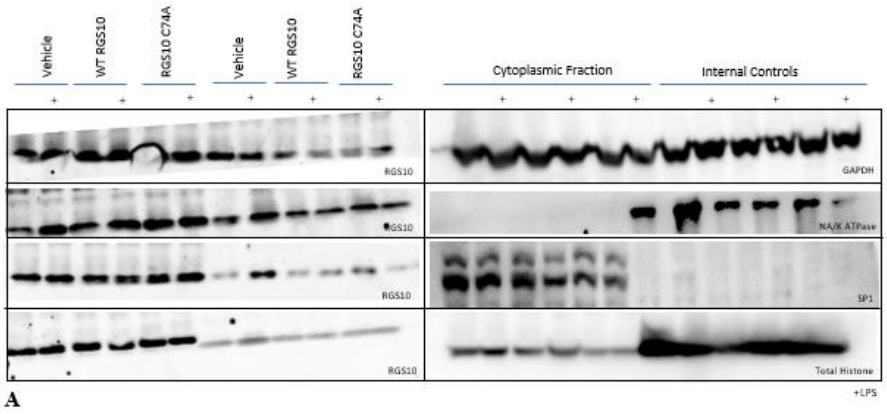

Figure 13.RGS10 in BV2 cells. Endogenous RGS10 is found in the cytoplasm, membrane and soluble fractions in BV2 cells. LPS treatment did not affect endogenous RGS10 translocation.

3.5. Effect of palmitoylation on RGS10 subcellular localization in HEK cells

HEK cells contain low endogenous levels of RGS10 making them an ideal vehicle for overexpression of wildtype RGS10 and RGS10 C74A. Two-hour treatment of LPS (10 µg/mL) and subsequent subcellular fractions revealed that endogenous, wild type RGS10 and RGS10 C74A are present in high level in the cytoplasm and lower levels in the membrane and soluble nuclear lysate. No RGS10 was found in the chromatin bound lysate. When treated with LPS, there was a higher expression of wild type RGS10 and RGS10 C74A in the membrane and soluble nuclear lysate indicating RGS10 possibly translocates to these areas (**Figure 14**). However, a comparison between the total RGS10 concentration in the cell and between each compartment could not be made. As a result, it could not be concluded that palmitoylation is involved in RGS10 translocation within the cell but seems very unlikely that this modification affects RGS10 translocation from the given results.

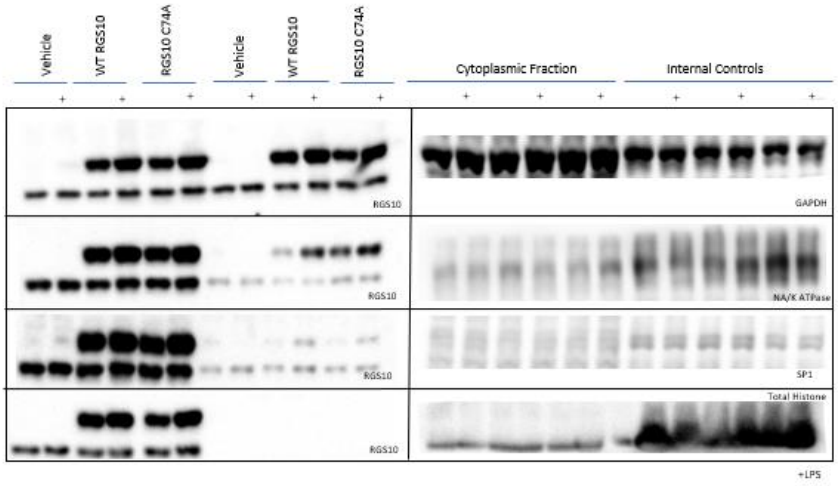

Figure 14. RGS10 in HEK cells. Endogenous and overexpressed RGS10 are found in the cytoplasm, membrane and soluble nucleus. Upon LPS stimulation, there is a higher expression in the membrane and soluble nuclear fraction in the wild type RGS10 and RGS10 C74A maybe indicating translocation**.**

3.6. Effect of palmitoylation on RGS10s ability to suppress TNFα

Lee et al (2008) demonstrated that RGS10 has the ability to suppress TNFα. To test whether RGS10 C74A retains the same properties, wildtype RGS10 and RGS10 C74A were transfected in HEK cells and treated with LPS for two hours. It was found that TNFα mRNA expression levels were not significantly different in RGS10 C74A in comparison to wildtype RGS10 after LPS treatment (**Fig 15A**). RGS10 mRNA transcription level was quantified as a measure of overexpression of the transfected vectors had occurred (**Fig 15B**). It was concluded that both wild-type RGS10 and RGS10 C74A are equally able to suppress TNFα transcription post LPS. Thus, palmitoylation does not play a role in RGS10's ability to regulate inflammatory signaling.

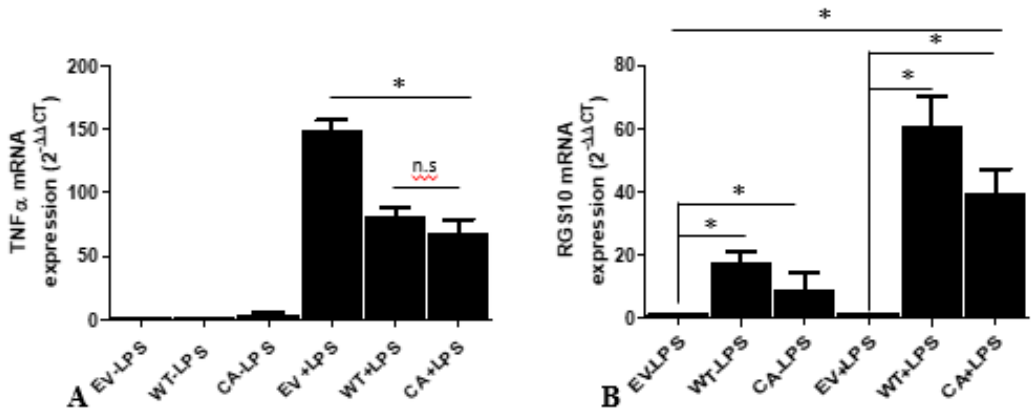

Figure 15. Effect of LPS on RGS10 and TNFα transcription. **A**. There is no significant difference in the RGS10's ability to suppress TNFα between the wild type and RGS10 C74A constructs. **B**. Significant overexpression of wild type (WT) RGS10 and RGS10 C74A was observed in comparison to empty vector (EV). Data were analyzed from three independent experiments and the difference between groups was analyzed by analysis of variance (ANOVA), followed by Bonferroni posttests. Data are presented as mean ± S.E.M., where $*P < 0.05$.

One intriguing find was the change in RGS10 overexpression in the presence of LPS. It was seen over three individual experiments that wild type RGS10 and RGS10 C74A constructs had a more robust overexpression level after LPS treatment (**Figure 15B)**. This was corroborated with western data comparing RGS10 with and without LPS treatment (Figure not shown). One explanation for this may be that there is a general overexpression of all proteins during a state of stress or inflammation. The difference in overexpression levels between wild type RGS10 and RGS10 C74A may be due to the quality of the plasmid. The RGS10 C74A construct may not be as pure as the wild type RGS10 construct, thus RGS10 C74A expression is constitutively lower.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Palmitoylation is a reversible post translational modification that has previously shown to aid in signal transduction [56], GPCR expression and function [52] and subcellular localization[57]. In RGS10, the role of palmitoylation is not fully understood. While some studies have found that palmitoylation negatively affects RSGS10 GAP function, others say it facilitates RGS10's GAP activity. Furthermore, the effect of palmitoylation on RGS10's subcellular localization has never been identified. Neither has the effect of palmitoylation in respect to RGS10 role in suppressing inflammation been studied. It has been shown that when stimulated with LPS, RGS10 has the ability to suppresses the inflammatory cytokine, TNFα (Lee et al, 2008). Thus, the aim of this study was to determine whether palmitoylation affects RGS10's subcellular localization or has any effect on its ability to suppress inflammation.

It was found that palmitoylation has no effect on RGS10 subcellular localization in either BV2 or HEK cells. It was also found that palmitoylation has no effect RGS10's ability to suppress TNFα in HEK cells. In fact, RGS10 C74A could suppress TNFα levels as well was wild-type RGS10.

However, it was found that LPS stimulation globally amplified protein expression in HEK cells. This was a recurrent pattern and worth further investigation. It was also found that under LPS stimulation, there was a higher expression of RGS10 at the membrane and soluble fraction in comparison to the non-stimulated group in both wild type RGS10 and RGS10 C74A. This could

indicate that LPS stimulation is prompting more RGS10 translocations to these subcellular compartments. A closer look at these subcellular compartments could shed light on more possible functions of RGS10. Furthermore, subcellular fractionation of the membrane isolates all cellular parts that contain membrane including the cell membrane, nuclear membrane and all membrane-bound organelles. Thus, it would be beneficial to identify which particular membrane RGS10 is localizing at after LPS stimulation to further understand the role of this protein.

Confocal microscopy and immunocytochemistry can further corroborate the subcellular fractionation results. And lastly, identifying the subcellular localization of other RGS10 constructs (i.e. GAP-dead RGS10) may shed light upon the non-canonical functions of RGS10 leading to a better understanding of how RGS10 is regulated in disease such as neuroinflammation.

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